The EMBO Journal vol.14 no.13 pp.3206–3214, 1995

Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*

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Agrobacterium tumefaciens **transfers part of its tumour-inducing (Ti) plasmid, the transferred or T-DNA, to plants during tumourigenesis. This represents the only example of naturally occurring trans-kingdom transfer of genetic material. Here we report that *A.tumefaciens* can transfer its T-DNA not only to plant cells, but also to another eukaryote, namely the yeast *Saccharomyces cerevisiae*. The Ti plasmid virulence (*vir*) genes that mediate T-DNA transfer to plants were found to be essential for transfer to yeast as well. Transgenic *S.cerevisiae* strains were analysed for their T-DNA content. Results showed that T-DNA circles were formed in yeast with precise fusions between the left and right borders. Such T-DNA circles were stably maintained by the yeast if the replicator from the yeast 2μ plasmid was present in the T-DNA. Integration of T-DNA in the *S.cerevisiae* genome was found to occur via homologous recombination. This contrasts with integration in the plant genome, where T-DNA integrates preferentially via illegitimate recombination. Our results thus suggest that the process of T-DNA integration is predominantly determined by host factors.**

Key words: *Agrobacterium tumefaciens*/homologous recombination/T-DNA transfer/virulence genes/yeast

Introduction

Agrobacterium tumefaciens is a Gram-negative soil bacterium able to induce tumours, or crown galls, at plant wound sites. During tumourigenesis part of its tumour-inducing (Ti) plasmid, the T-DNA, can be mobilized from the bacteria into the plant cell by the virulence (*vir*) genes located on the Ti plasmid. Expression of *onc* genes on the T-DNA leads to plant cell proliferation and formation of a tumour. *vir*-mediated plant cell transformation represents the only example of naturally occurring trans-kingdom DNA transfer.

vir genes involved in T-DNA transfer have been extensively studied (for reviews see Hooykaas and Schilperoort, 1992; Winans, 1992; Zambryski, 1992; Hooykaas and Beijersbergen, 1994). The transfer system is activated when VirA senses inducing compounds produced at the plant wound sites, such as acetosyringone (AS), and activates the remaining *vir* loci via the transcriptional activator VirG. Upon activation of *vir* gene expression a linear single-stranded DNA, the T-strand, is generated following nicking of the border repeats surrounding the T region (Albright *et al.*, 1987; Wang *et al.*, 1987). The border repeats are nicked by the VirD2 protein with help from the VirD1 protein (Lessl and Lanka, 1994). The VirC proteins increase the efficiency of the right border (RB) nicking reaction by binding to an enhancer sequence located next to the RB (Toro *et al.*, 1988) and in this way can affect host range. Nicking leads to covalent attachment of the VirD2 protein to the 5′-end of the T-DNA. The C-terminal part of VirD2 contains nuclear localization sequences (NLSs) for transport of the T-DNA to the nucleus (Tinland *et al.*, 1992; Rossi *et al.*, 1993). The T-DNA is transferred to the plant cell as a single-stranded molecule (Chaudhury *et al.*, 1994; Tinland *et al.*, 1994; Yusibov *et al.*, 1994). Also transferred to the plant cell is the VirE2 protein, a single-stranded DNA binding protein which may coat the T-DNA along its length to form long nucleoprotein filaments (Citovsky *et al.*, 1989). It has been proposed that transferred *vir* proteins may promote T-DNA integration in the genome of the plant cell. T-DNA is thought to leave the *Agrobacterium* cell through a transmembrane structure consisting of products of the *virB* operon. Most of the 11 VirB proteins are located in the membrane (Beijersbergen *et al.*, 1994) and, with the exception of the VirB1 protein, are all essential for tumourigenesis (Berger and Christie, 1994). T-DNA transfer to certain plant species also requires the host range proteins VirF and VirH. The precise function of these is unknown. The VirH protein shares some homology with the P450 cytochrome class of enzymes and therefore may play a role in detoxification of plant exudate products (Kanemoto *et al.*, 1989). VirF is probably transported to the plant cell and is necessary for T-DNA transport to some plant species (Regensburg-Tuink and Hooykaas, 1993).

Agrobacterium tumefaciens induces tumours in a wide range of dicotyledonous plant species, but not in monocotyledonous plants. Nevertheless, T-DNA transfer to monocots could be demonstrated indirectly by the use of sensitive reporter systems, such as agroinfection (Grimsley *et al.*, 1987). Recently Hiei *et al.* (1994) used the *Agrobacterium* vector system to obtain transgenic rice plants. Subsequently it was found that T-DNA integrated into the genome of rice in the same way as in dicots.

We were interested to see whether T-DNA transfer and integration would be possible to species belonging to another kingdom than that of plants. We chose the yeast *Saccharomyces cerevisiae* (kingdom Fungi) because of its

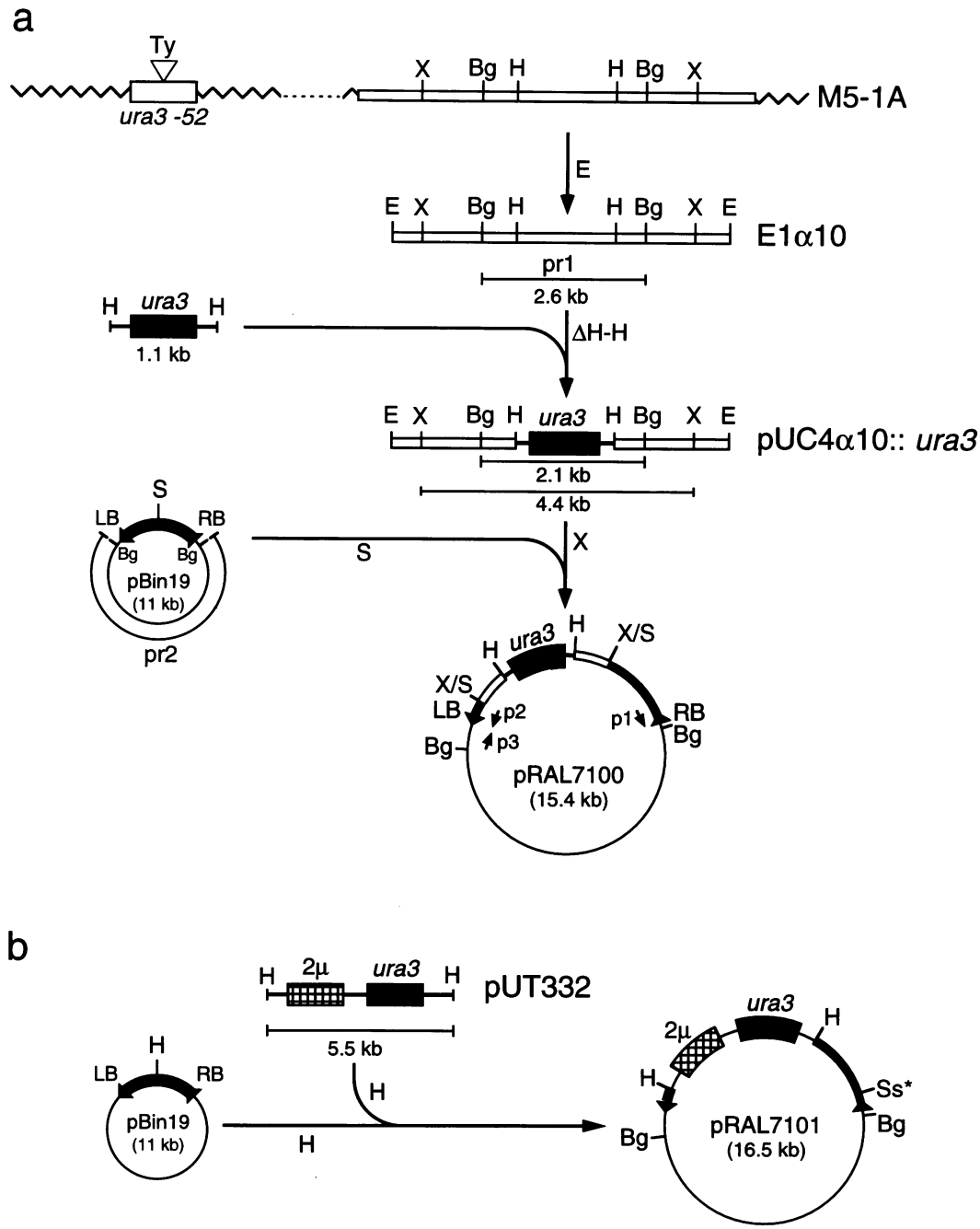

Fig. 1. Construction of the 'integrative' plasmid pRAL7100 (**a**) and 'replicative' plasmid pRAL7101 (**b**). The wild-type chromosomal *PDA1* locus of M5-1a is located between the *Hin*dIII sites. Restriction enzymes: Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Ss, *Sst*II; X, *Xho*I. ΔH-H, deletion of *Hin*dIII–*Hin*dIII *PDA1* fragment from clone E1α10 (Steensma *et al.*, 1990). Ty, transposon Ty; pr1, probe 1; pr2, probe 2; p1–p3, primers 1–3; LB, left border repeat; RB, right border repeat; *, no unique restriction site (not drawn to scale).

ease of handling, the availability of suitable vectors and its fast growth rate. The results of these studies are described in this article. We discovered that T-DNA transfer to *S.cerevisiae* is possible, suggesting that in nature the host range of *Agrobacterium* may be even broader than so far anticipated. We found that *vir* genes required for T-DNA transfer to plants were also necessary for T-DNA transfer to yeast. T-DNA was integrated via homologous recombination in the yeast genome. Therefore the process of T-DNA integration seems to be largely determined by host factors.

Results

T-DNA transfer from A.tumefaciens to S.cerevisiae

Transformation of yeast with a replicative vector is much more efficient than with an integrative vector. Therefore, in order to establish whether *A.tumefaciens* could transfer T-DNA to the yeast *S.cerevisiae*, we constructed the binary vector pRAL7101. This plasmid contains the yeast *URA3* selection gene (Rose *et al.*, 1984) and the yeast 2μ origin of replication between the left border (LB) and RB repeats of the binary vector pBIN19 (Figure 1b). To be able to

Table I. T-DNA transfer to *Saccharomyces cerevisiae* strain M5-1a

Plasmid present in donor *Agrobacterium* strain LBA1100	Medium	M5-1a colonies on medium without uracil	M5-1a colonies (10^8/ml) on medium with uracil	Frequency of Ura^+ colonies per output recipient
pRAL7100	+AS	272	1.6	1.7×10^{-6}
	−AS	0	0.2	$<5\ \times10^{-8}$
pRAL7101	+AS	200	0.6	3.3×10^{-6}
	−AS	0	2.2	$<4.5\times10^{-9}$

test for transfer, pRAL7101 was electroporated into the *A.tumefaciens* helper strain LBA1100, which carries the *vir* genes that code for the T-DNA transfer system (Beijersbergen *et al.*, 1992). Activation of the *vir* genes can be accomplished *in vitro* by incubating the strain in a low pH medium containing the phenolic inducer AS (Scheeren-Groot *et al.*, 1994). Incubation of *S.cerevisiae* in this medium did not inhibit growth or lead to lethality and mixtures of agrobacteria and yeast cells also survived provided that the glucose concentration was <5 mM. After these control experiments, co-cultivations between *A.tumefaciens* and *S.cerevisiae* strain M5-1a, which is a haploid Ura^- strain, were carried out to test for T-DNA transfer. After incubation on low pH medium with or without AS (as a control), cells were resuspended in 0.9% NaCl and plated onto selective media. As can be seen in Table I, URA3 transfer from *A.tumefaciens* to *S.cerevisiae* was indeed observed and was dependent on the presence of the *vir* inducer AS in the medium. The Ura^+ yeast strains thus obtained were purified and then characterized.

The Ura^+ phenotype turned out to be stable. There was no loss of marker after overnight growth in non-selective medium. To assay for the presence of T-DNA, a simple DNA preparation was carried out on these Ura^+ strains. Since we suspected that a plasmid replicating via the 2μ replicator was present, the isolated DNA was used for transformation of *Escherichia coli*, from which DNA can be purified more easily than from yeast. Selection was for carbenicillin resistance (Cb^r), a selective marker that was present in the T-region of pRAL7101, together with the ColE1 origin of replication and the *URA3* gene. DNA preparations from all of the purified Ura^+ yeast strains resulted in Cb^r *E.coli* transformants. Forty-eight *S.cerevisiae* strains were analysed for their T-DNA content in this way.

Three of the 48 strains contained a complete circular T-DNA and their restriction patterns confirmed that the two *Bgl*II sites present on either side of the borders of pRAL7101 had been lost, while the *Sst*II site (Figure 1b) next to the RB was still present (data not shown). We concluded that in these co-cultivations transfer of a T-strand to M5-1a had occurred. T-DNA is transferred as a linear single-stranded molecule (Chaudhury *et al.*, 1994; Tinland *et al.*, 1994; Yusibov *et al.*, 1994) and therefore a ligation step is required for the formation of a plasmid in yeast. Plasmids from these strains were tested with primers p1 and p2, which flank the border repeats of the T region. All three strains amplified a 703 bp fragment consistent with a border fusion (Figure 2a, lane 4). The PCR product was sequenced and a comparison of sequences surrounding the T-region revealed that the T-circles of these three strains each contained one intact border repeat. The sequence of this border repeat was

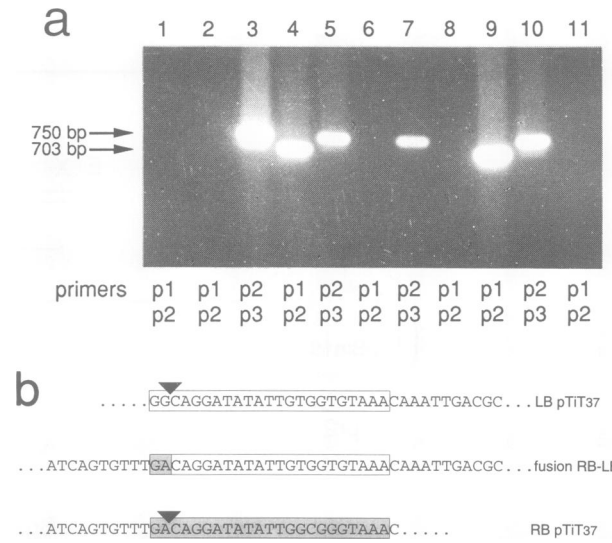

Fig. 2. (**a**) PCR analysis of Ura^+ transgenic *S.cerevisiae* strains after T-DNA transfer from LBA1100 (pRAL7101) (lanes 2–5) or LBA1100 (pRAL7100) (lanes 6–10). Primers used are indicated as p1–p3 (see also Figure 1). The size of the products (in base pairs) is indicated by arrows. Lane 1, M5-1a; lane 2, pRAL7101 (plasmid control, 100 pg); lane 3, pRAL7101 (plasmid control, 100 pg); lane 4, pRAL7101 T-region transferred; lane 5, entire pRAL7101 transferred; lane 6, pRAL7100 (plasmid control, 100 pg); lane 7, pRAL7100 (plasmid control, 100 pg); lane 8, double cross-over between the T-region of pRAL7100 and the yeast *PDA1* locus; lane 9, M5-1a containing a circularized T-strand derived from pRAL7100; lane 10, integration of entire pRAL7100 plasmid; lane 11, H_2O control. (**b**) Comparison of the nucleotide sequences of the LB and RB repeats with the fused border sequence found in the transferred T-DNAs. pBIN19 border sequences are derived from the wild-type nopaline plasmid pTiT37 (Bevan, 1984). The boxed areas indicate the border repeats; ▼, nick site, established *in vivo*, *in vitro* and corresponding to homology with the RP4 nick site (Lessl and Lanka, 1991).

identical for all three strains and was compatible with a precise fusion between processed RB and LB repeats, i.e. the first 2 nt derived from the RB and the remaining 22 nt from the LB (Figures 2b and 4a).

Sixteen of the Ura^+ M5-1a colonies contained a plasmid smaller than expected if T-strand transfer and circularization had occurred and did not amplify a fragment during PCR with primers p1 and p2. In order to characterize these plasmids DNA sequencing was performed. In these 16 strains the plasmid formed was the result of homologous recombination between a direct repeat consisting of part (150 bp) of the bleomycin gene. This 150 bp sequence turned out to be accidently present between the borders of the original pBIN19 vector and also on the introduced pUT332 fragment (Mazodier *et al.*, 1985). Therefore, these small T-circles were formed by intra-T-DNA recombination after transfer, resulting in deletion of the border sequences.

Table II. No transformation of M5-1a after addition of plasmid DNA to the donor–recipient mixtures

Plasmid DNA added	No. of M5-1a colonies on medium without uracil	No. of M5-1a colonies (10^7/ml) on medium with uracil	Transformation frequency
pRAL7100[a]	0	4.2	$<2.4\times10^{-8}$
pRAL7100[b]	0	6.4	$<1.6\times10^{-8}$
pRAL7101[a]	0	8.4	$<1.2\times10^{-8}$
pRAL7101[b]	0	4.2	$<2.4\times10^{-8}$

[a]Addition of 1 μg DNA to the co-cultivation medium.
[b]Addition of 5 μg DNA to the co-cultivation medium.
Strain LBA1100 (lacking the T-DNA *in trans*) was used as the donor.

Surprisingly, plasmids from the remaining 29 Ura$^+$ yeast strains showed restriction patterns which indicated that they contained the entire pRAL7101 plasmid. It can be argued that in these cases T-DNA transfer may not have occurred, but rather that these strains may have been generated by uptake of intact plasmid DNA present in the medium. However, in a model transfer experiment (Table II), no Ura$^+$ *S.cerevisiae* strains were found when 1 or 5 μg plasmid DNA (pRAL7101 or pRAL7100) was added to a mixture of donor (LBA1100 lacking the T-region *in trans*) and acceptor cells (M5-1a). Also, transfer from *Agrobacterium* was unaffected by addition of 1 μg/ml DNase I to the incubation medium, showing that the T-DNA is protected against this enzyme during transfer. Therefore, the presence of the whole pRAL7101 plasmid in a large number of the yeast Ura$^+$ strains can be best explained by missing of the LB, which was not nicked during T-DNA processing in the bacterial cell. This has been shown to occur during *in vitro* T-DNA processing experiments (Stachel *et al.*, 1987), as well as in T-DNA transfer to plants, where 20% of transformed plants were found to contain DNA sequences present outside the T-DNA borders (Martineau *et al.*, 1994). It was confirmed that the Ura$^+$ yeast strains which showed the entire restriction pattern of pRAL7101 contained an intact LB, as shown by amplification of a 750 bp fragment using primers p2 and p3, which were located on either side of the LB (Figure 1 and Figure 2a, lane 5). Missing of the LB, transfer of the entire plasmid and ligation at the RB would be expected to re-create the original binary vector in *S.cerevisiae*.

Evidence for T-DNA integration into the yeast genome

In plant species (T-)DNA integration occurs via illegitimate recombination (Offringa *et al.*, 1990), but DNA introduced into *S.cerevisiae* integrates into the genome predominantly via homologous recombination. We were interested in whether T-DNA carrying extensive homology with the *S.cerevisiae* genome would integrate via homologous recombination or illegitimate recombination. Therefore, an 'integrative' binary vector, pRAL7100, was constructed with the *URA3* selection gene surrounded by DNA derived from the flanking regions of the *S.cerevisiae PDA1* gene (Figure 1a), which could promote homologous recombination between the introduced T-DNA and the *S.cerevisiae PDA1* locus on chromosome V. The T-DNA of pRAL7100 carries no yeast origin of replication. Therefore, growth of

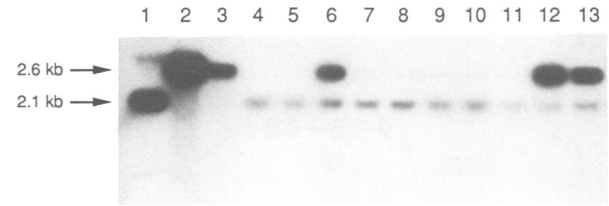

Fig. 3. Autoradiograph of a Southern blot of Ura$^+$ transgenic *S.cerevisiae* strains obtained after transfer of DNA from LBA1100 (pRAL7100) (see Materials and methods). Total DNA digested with *Bgl*II was hybridized with the 2.6 kb *Bgl*II fragment from clone E1α10 (probe pr1, Figure 1a). The fragment sizes (in kilobase pairs) are indicted by arrows. Lane 1, pRAL7100 (0.1 μg); lane 2, E1α10 (0.1 μg); lane 3, M5-1a; lanes 4–13, Ura$^+$ yeast strains. Lanes 4, 5 and 7–11 lack the 2.6 kb band, indicating a gene replacement event had occurred. Lanes 6, 12 and 13 represent Ura$^+$ M5-1a strains in which the whole pRAL7100 plasmid had integrated into the genome via a single cross-over.

S.cerevisiae on medium lacking uracil after co-cultivation with *A.tumefaciens* should be due to integration of the T-DNA carrying the *URA3* marker into the yeast genome.

Ura$^+$ M5-1a colonies were indeed found after co-cultivation with LBA1100 (pRAL7100) on plates containing AS (Table I) and, surprisingly, at approximately the same frequency as after co-cultivation using LBA1100 (pRAL7101) as the donor. This result was unexpected, because the replicating vector pRAL7101 gave an ~100-fold higher frequency of Ura$^+$ M5-1a transformants than the integrative vector pRAL7100 after electroporation (data not shown).

Twenty of the Ura$^+$ yeast strains generated after co-cultivation with *A.tumefaciens* containing pRAL7100 were purified. Total DNA was isolated from these and a Southern blot was carried out to detect the presence of integrated T-DNA. Using a 2.6 kb *Bgl*II fragment from the *PDA1* gene as a probe (Figure 1a, probe pr1), the same 2.6 kb *Bgl*II fragment was expected in the wild-type and in strains still containing an unmodified *PDA1* gene. However, a 2.1 kb *Bgl*II fragment was expected for strains containing the modified *PDA1* gene with an integrated *URA3* gene. In the DNA of 12 of the Ura$^+$ yeast strains only a 2.1 kb homologous fragment was detected (Figure 3). In these strains, apparently, the incoming T-DNA with the modified *PDA1* gene had recombined with the homologous *PDA1* locus and introduced the *URA3* gene via a double cross-over or gene conversion (Figure 4c). The remaining eight strains still contained the 2.6 kb wild-type *PDA1* fragment, in addition to the 2.1 kb fragment. This is consistent with a single cross-over event having occurred between one of the *PDA1* flanking sequences present on the incoming T-DNA and the homologous sequence in the yeast genome. To find further evidence for this, DNA from the eight Ura$^+$ strains were digested with *Eco*RI and probed with pr3 (a 0.5 kb *Eco*RI–*Xho*I fragment of the *PDA1* gene). A single cross-over on either side of the *PDA1* locus would be expected to give a band of 16.5 kb with this probe and indeed in three of the strains the 16.5 kb band was detected (data not shown). All three strains showing this 16.5 kb band also amplifed the 750 bp fragment with primers p2 and p3 (Figure 2a, lane 10), showing that they all contain an integrated complete binary vector. Together, these data suggest that these strains were formed after

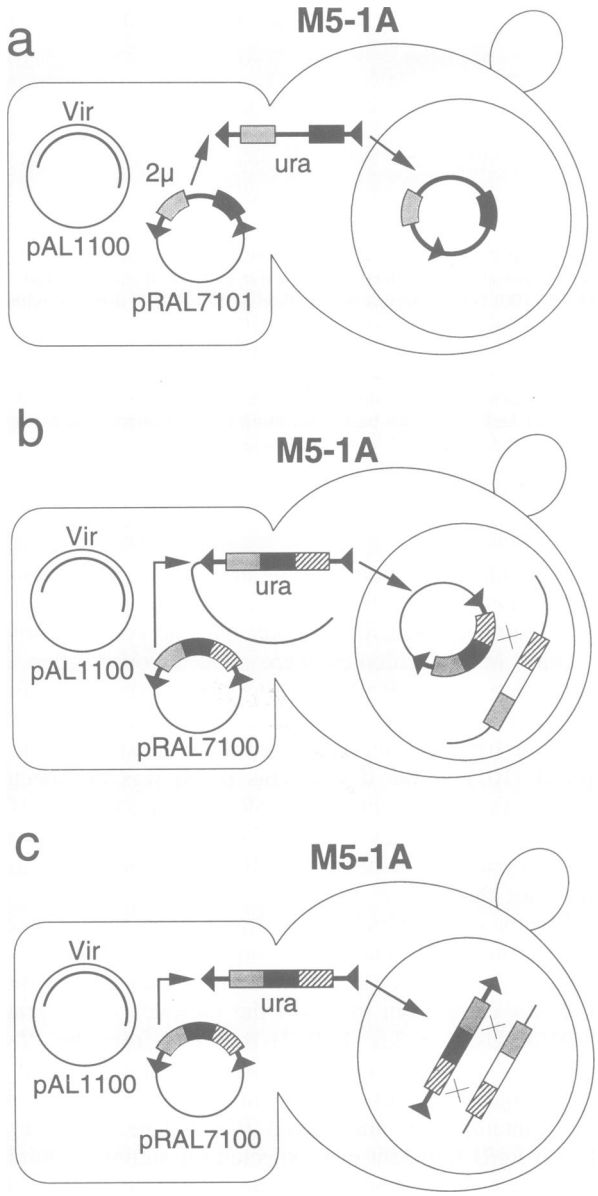

Fig. 4. Representation of the different T-DNA transfer events from *A.tumefaciens* to *S.cerevisiae* using plasmid pRAL7101 (**a**) or pRAL7100 (**b** and **c**). An *Agrobacterium* cell is shown on the left and a *Saccharomyces* cell on the right. Transgenic *S.cerevisiae* strains were formed by (a) autonomous replication of the transferred T-DNA, (b) integration of pRAL7101 via a single cross-over and (c) integration via a double cross-over. In (a) only T-DNA is transferred; in 60% of the cases we found transfer of the entire pRAL7101.

missing of pRAL7100 LB during T-DNA processing, transfer of the whole pRAL7100 plasmid and subsequent circularization and integration of the whole pRAL7100 via a single cross-over event at the *PDA1* locus.

In the remaining five strains no evidence of homologous recombination between the *PDA1* locus and the T-DNA could be seen. We therefore attempted to localize the T-DNA insertions in these strains by separating the yeast chromosomes on a CHEF gel and blotting with a 300 bp *PstI–SphI* fragment of the T-DNA as a probe. No signal on any chromosome could be detected, whereas in controls the T-DNA integrated by a single cross-over could be localized to chromosome V, which carries the *PDA1* locus.

S.cerevisiae itself is able to ligate introduced DNA to mitochondrial sequences (Schiestl *et al.*, 1994) and this could also account for the as yet unidentified forms of T-DNA in these strains.

Effect of vir mutations on the transfer of T-DNA to S.cerevisiae

To define the Vir functions necessary for T-DNA transfer to *S.cerevisiae*, the binary vectors pRAL7100 and pRAL7101 were electroporated into a series of *A.tumefaciens* strains containing mutations in the *vir* genes. Co-cultivations with the *A.tumefaciens* mutants and *S.cerevisiae* strain M5-1a were performed as in the previous experiments and the results are summarized in Table III.

All mutants tested showed decreased or zero T-DNA transfer as compared with the wild-type, except for the *virF* mutant. Mutations in the *virA* and *virG* genes prevented transfer as expected, as these two Vir proteins are essential for induction of the remaining *vir* genes. No Ura^+ M5-1a strains were produced by co-cultivation with the *virD4* or *virB* mutants, supporting the proposed role of these proteins in transfer of the T-strand. No Ura^+ M5-1a colonies were produced during co-cultivation with a strain carrying a mutation in the 3′-region of *virD2*. This particular mutation does not affect the nicking function or *vir*-mediated bacterial conjugation (Beijersbergen *et al.*, 1992), but deletes the NLS sequences and the proposed omega sequence, which are important for T-DNA transfer to plants (Shurvinton *et al.*, 1992). Apparently this 3′-region of VirD2 is also important for transfer to yeast. The *virE2* mutant showed a much reduced transfer frequency. The VirE2 protein is important, but not absolutely essential, for T-DNA transfer to plants (Yusibov *et al.*, 1994), however, it plays no role in *vir*-mediated bacterial conjugation (Beijersbergen *et al.*, 1992). The VirE2 protein was reported to be able to coat T-strands and thus be able to protect the T-DNA against nucleases present in eukaryotic cytosol. Since the absence of VirE2 might lead to the generation of deletions in the T-DNA, autonomously replicating plasmids were isolated from Ura^+ M5-1a strains obtained after co-cultivation with the *virE2* mutant containing pRAL7101. The same three plasmid types were isolated as were previously obtained when wild-type LBA1100 (pRAL7101) was used in co-cultivations (see above). Therefore, the lack of VirE2 protein did not lead to any apparent deletions during transfer of the T-DNA to yeast. However, it is possible that in the absence of VirE2 nuclease action digests 3′ sequences necessary for T-DNA circularization in yeast. This could account for the observed drop in frequency for all three plasmid types. Alternatively, the reduction in frequency seen after co-cultivation of yeast with the *virE2* mutant may be due to the loss of the nuclear targeting function mediated by the NLS present on the VirE2 protein (Citovsky *et al.*, 1992). Also, in the absence of VirE2, the T-DNA may be unable to form a long unfolded T-complex, which seems necessary for efficient T-DNA transport (Citovsky *et al.*, 1989).

The *virF* mutant was the only *A.tumefaciens* strain which transferred T-DNA at a frequency equivalent to the wild-type strain during co-cultivations. It is thought that the *virF* protein influences the host range of *A.tumefaciens* by an interaction with a structure or protein in the plant cell (Jarchow *et al.*, 1991; Regensburg-Tuink and

Table III. T-DNA transfer from *A.tumefaciens* donor strains to the *S.cerevisiae* recipient haploid strain M5-1a

Mutant donor	*vir* mutation	Plasmid	Titre input (10^7 cells/ml)		Titre output (10^7 cells/ml)		No. of M5-1a colonies on medium without uracil	Frequency of Ura^+ colonies per output recipient
			Donor	Recipient	Donor	Recipient		
LBA1100		pRAL7101	1.8	2.0	24.0	6.0	200	3.3×10^{-6}
		pRAL7100	1.9	2.0	34.0	16.0	272	1.7×10^{-6}
LBA1142	*vir*A	pRAL7101	5.0	2.3	2.0	9.0	0	$<1.1\times10^{-8}$
		pRAL7100	3.0	1.5	7.0	8.0	0	$<1.2\times10^{-8}$
LBA1143	*vir*B4	pRAL7101	2.2	1.9	1.0	31.0	0	$<3.2\times10^{-9}$
		pRAL7100	2.4	1.6	3.0	5.0	0	$<2\times10^{-8}$
LBA1144	*vir*B7	pRAL7101	4.6	1.9	1.5	7.0	0	$<1\times10^{-8}$
		pRAL7100	4.4	2.0	1.0	11.0	0	$<9\times10^{-9}$
LBA1145	*vir*G	pRAL7101	5.6	1.9	2.0	15.0	0	$<6.6\times10^{-9}$
		pRAL7100	5.6	1.5	8.0	9.0	0	$<1\times10^{-8}$
LBA1147	3′*vir*D2	pRAL7101	4.1	2.1	4.5	13.0	0	$<7.7\times10^{-9}$
		pRAL7100	4.8	1.5	6.0	13.0	0	$<7.7\times10^{-9}$
LBA1148	*vir*D4	pRAL7101	2.0	2.1	3.2	16.0	0	$<6.3\times10^{-9}$
		pRAL7100	3.0	1.5	6.5	7.0	0	$<1.4\times10^{-8}$
LBA1149	*vir*E2	pRAL7101	3.0	2.0	24.0	6.0	18	3×10^{-7}
		pRAL7100	2.4	2.0	6.0	14.0	24	1.7×10^{-7}
LBA1517	*vir*F	pRAL7101	3.7	2.0	3.0	6.0	231	3.9×10^{-6}
		pRAL7100	2.7	2.0	4.5	11.0	233	2.1×10^{-6}

Each *A.tumefaciens* mutant carried either pRAL7101 (replicative vector) or pRAL7100 (integrative vector). AS (200 μM) was included in the co-cultivation plates. Similar results were obtained from at least three independent experiments. The numbers of donor and recipient cells were determined at mixing (titre input) and after 3 days co-cultivation (titre output).

Hooykaas, 1993). Apparently, this interacting structure or protein is absent from the yeast cell. Plasmids isolated from Ura^+ M5-1a strains generated by co-cultivation with the *virF* mutant were also detected in the three forms described when LBA1100 (pRAL7101) was used as a donor.

Discussion

In this report we show for the first time that Ura^- *S.cerevisiae* cells can be converted to Ura^+ by incubation with an *Agrobacterium* donor containing a binary vector with the *URA3* gene. The transferred DNA was found to be protected against nucleases in the medium during transfer. In contrast, plasmid DNA added to an artificial co-cultivation mixture of agrobacteria and yeast cells was not taken up by *S.cerevisiae*. Transfer occurred via an active process mediated by the *Agrobacterium vir* system, as shown by the essential role of the *vir* genes, suggesting that T-DNA transfer to yeast and plants occurs via a common mechanism. Together, these data show that the host range for *Agrobacterium* T-DNA transfer includes yeasts, in addition to plants.

DNA transfer to *S.cerevisiae* has also been reported from *E.coli* containing a conjugative IncP plasmid (Heinemann and Sprague, 1989) or a mobilizable IncQ plasmid (Nishikawa *et al.*, 1992). It has been proposed that the *tra* system of certain conjugative plasmids and the *vir* system of Ti share common evolutionary origins. This idea has been supported by studies showing IncQ transfer from *A.tumefaciens* to plants (Buchanan-Wollaston *et al.*, 1987) and to other bacteria (Beijersbergen *et al.*, 1992) mediated by the *vir* system. Homology studies have found significant similarities between the processing and transport proteins of the *tra* and *vir* systems (reviewed in Lessl and Lanka, 1994) and between the nick regions of a variety of DNA transfer systems (Pansegrau and Lanka, 1991). Our experiments support the proposed link between the *vir* system and other DNA transfer systems, although *Agrobacterium* and its *vir* system seem optimized for DNA transfer to plant species.

Surprisingly, in our co-cultivation experiments with *Agrobacterium*, the frequencies with which Ura^+ *S.cerevisiae* strains were obtained were similar for the replicating vector (pRAL7101) and the integration vector (pRAL7100). In electroporation experiments, as expected, the replicating vector pRAL7101 was more efficient than the integration vector pRAL7100. This difference in results between co-cultivation and electroporation might be due to the different DNA structures that are introduced into yeast, single-stranded linear DNA during the T-DNA transfer process and double-stranded circular DNA during electroporation. However, when RSF1010-derived vectors containing the *URA3* gene were mobilized from *E.coli* into *S.cerevisiae* (Nishikawa *et al.*, 1992), Ura^+ *S.cerevisiae* colonies were obtained at a higher frequency with an ARS-type replicative vector compared with an integrative vector. In this latter case the DNA is probably introduced in a single-stranded linear form, like T-DNA. Since the yeast host factors in IncQ and T-DNA transfer are the same, the abnormal behaviour of the T-DNA transfer system in yeast must be due to the Vir proteins that accompany the T-DNA. It can be envisaged that these stimulate T-DNA integration, making integrative T-DNAs equally efficient as replicating T-DNAs. We think this unlikely, because in plants T-DNA is integrated in a manner strikingly different from that in yeast, i.e. illegitimate versus homologous recombination (see below). Alternatively, it can be argued that replicative T-DNAs have difficulty in establishing themselves in yeast. An interesting possibility could be that the VirD2 pilot protein, which shows *in vitro* nickase/ligase activity (Pansegrau *et al.*, 1993a, 1994), is inefficient in circularizing the T-strand. After all, in plants circularization does not normally

occur and the ends of the T-DNA are used for integration (Mayerhofer *et al.*, 1991). The VirD2 protein may be different in its poor ligase activity from the functionally homologous nickase/ligase protein MobA encoded by the IncQ plasmid (Scherzinger *et al.*, 1992) and the TraI protein encoded by the IncP plasmid (Pansegrau *et al.*, 1993b). Work in our laboratory is in progress to find out whether *vir*-mediated IncQ plasmid transfer to yeast is different for integration and replication vectors.

We were surprised to find a large number of Ura^+ yeast strains containing all of the pRAL7101 binary vector DNA instead of only the T-DNA. Apparently, during T-DNA processing the LB is missed quite often. This seems to be typical for binary vectors and is in contrast to processing of the wild-type Ti plasmid. Unequivocal evidence for missing of the LB of the wild-type Ti has never been given, although many tumour lines were analysed for their T-DNA content. However, a large number of transgenic plants transformed with a binary vector were recently reported to contain sequences from outside the T-DNA borders (Martineau *et al.*, 1994). If transfer of the whole binary vector to yeast is common, it might be argued that the T-circles observed in yeast originate from these whole circles by homologous recombination on the 24 bp border repeats. There are three arguments against this reasoning. First, we have not observed instability in the pRAL7101 plasmid maintained by yeast, suggesting that recombination must take place early in establishment. Second, the frequency of recombination between direct repeats in yeast shows a linear dependence on the length of the DNA homology (Jinks-Robertson *et al.*, 1993), the minimal length necessary for homologous recombination being ~270 bp. This would make the 24 bp border repeats unlikely targets for homologous recombination. Third, if homologous recombination occurred between the border repeats then a cross-over may occur anywhere within this repeat. However, in all cases analysed by sequencing we found that the result was compatible with border ligation, whereas homologous recombination may equally well result in a product that is incompatible with border ligation. For these three reasons we favour the idea that the T-DNA circles found in yeast are formed via circularization of the T-DNA. Differences between recombination enzymes and nucleases in plants and yeast may explain why exact T-DNA border fusions were not recovered from plants, not even when these events were selected for (Bakkeren *et al.*, 1989). T-circles found in bacteria were the result of recombination between the border repeats (Koukolíková-Nicola *et al.*, 1985), but here this was induced by the nicking of these border repeats via the *vir* system (Timmerman *et al.*, 1988).

Plants integrate incoming (T-)DNA into the genome preferentially by illegitimate recombination; homologous recombination has only been shown to occur at low frequencies, when the T-DNA shares extensive homology with the target locus (Offringa *et al.*, 1990). However, in lower eukaryotes such as *S.cerevisiae*, integration of the introduced DNA occurs predominantly via homologous recombination (Hinnen *et al.*, 1978). We have demonstrated in this paper that T-DNA is also integrated in this latter way in *S.cerevisiae*, showing that the host proteins, rather than the *Agrobacterium vir* proteins, are the decisive factors in the integration process.

T-DNA transfer to *S.cerevisiae* can be used as a powerful tool to study the interaction of *A.tumefaciens* with eukaryotic recipient cells and to study the influence of different recombination systems on T-DNA integration. From our experiments we suggest that trans-kingdom DNA transfer may be more widespread than previously thought. When the Vir system is activated by plant wound products, *Agrobacterium*-mediated T-DNA transfer might be possible to a wide range of recipients, including fungal and maybe even certain animal cells. This system may therefore be useful in the genetic modification of species that are so far recalcitrant to transformation. However, in nature the correct conditions necessary for activation of the Vir system will only be available in the vicinity of plants, thus making natural T-DNA transfer to species other than plants most unlikely.

Materials and methods

Agrobacterium and Saccharomyces strains

The *Agrobacterium* strains used are listed in the Table IV (Beijersbergen *et al.*, 1992; Regensburg-Tuink and Hooykaas, 1992). *Saccharomyces* strain M5-1a (MATa *trp1-92 leu2-3/112 ura3-52 his4*) was used as the recipient. Cloning was done in *E.coli* strain MH1.

Table IV. *Agrobacterium* strains used

Strain	Chromosomal background	Plasmid
LBA1100	C58	pAL1100ΔT-DNA, Δ*tra*, Δ*occ*
LBA1142	C58	pAL1100(*virA*::Tn*3*HoHo)
LBA1143	C58	pAL1100(*virB4*::Tn*3*HoHo)
LBA1144	C58	pAL1100(*virB7*::Tn*3*HoHo)
LBA1145	C58	pAL1100(*virG*::Tn*3*HoHo)
LBA1147	C58	pAL1100(3′*virD2*::Tn*3*HoHo)
LBA1148	C58	pAL1100(*virD4*::Tn*3*HoHo)
LBA1149	C58	pAL1100(*virE2*::Tn*3*HoHo)
LBA1517	C58	pTiB6(*virF*::Tn*1831*)

Plasmid constructions

Plasmid pRAL7100 was constructed by the ligation of a 4.4 kb *Xho*I–*Xho*I fragment of pUC4α10::*ura3* (Steensma *et al.*, 1990) to the *Sal*I-digested vector pBIN19 (Bevan, 1984). Construct pRAL7101 was made by ligation of *Hin*dIII-digested DNA of plasmid pUT332 (Gaitignol *et al.*, 1990) to pBIN19 digested with *Hin*dIII. *Agrobacterium* strains were electroporated with these constructs as described by Mozo and Hooykaas (1991).

T-DNA transfer experiments

Agrobacterium strains containing the binary vector pRAL7100 or pRAL7101 were grown at 29°C overnight in minimal medium (Hooykaas *et al.*, 1979) containing the appropriate antibiotics at the following concentrations: kanamycin, 100 μg/ml; streptomycin, 250 μg/ml; carbenicillin, 75 μg/ml. The *Saccharomyces* recipient strains were grown overnight at 30°C in CY medium (Sherman *et al.*, 1983). After dilution of the *Agrobacterium* cells to an $OD_{660\ nm} \approx 0.15$ in induction medium [IM; composed of MM salts and 40 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.3, 10 mM glucose, 0.5% (w/v) glycerol and 200 μM AS] the *Saccharomyces* cells were diluted 10 times in fresh CY medium and the cultures were subsequently grown for 6 h at 29 and 30°C respectively. The input number of *Agrobacterium* and *Saccharomyces* cells was accurately determined by plating out dilutions of the cultures: for *Agrobacterium* on LC containing rifampicin (20 μg/ml) and for *Saccharomyces* on MY containing the required amino acids. Subsequently, 50 μl of both the *Agrobacterium* and *Saccharomyces* cultures were mixed and placed on 0.45 μm cellulose nitrate filters on IM plates containing 5 mM glucose and the amino acids histidine, tryptophan

(20 μg/ml) and leucine and uracil (30 μg/ml). After incubation for 3 days at 29°C the co-cultivation mixture was then resuspended in 2 ml of a physiological salt solution (PZ; 9 g NaCl/l) and 100 μl aliquots of this mix were plated. Ura^+ M5-1a strains were selected for on MY medium (Zonneveld, 1986) containing 200 μM cefotaxim to kill the *Agrobacterium* cells. The number of surviving *Agrobacterium* cells was determined by plating on LC plus rifampicin and the number of M5-1a cells was determined by plating dilutions on MY containing the required amino acids, including uracil.

DNA isolation from Saccharomyces strains

Chromosomal DNA was isolated from a 100 ml culture using the method decribed by Holm *et al.* (1986). DNA (5 μg) was digested with *Bgl*II for 16 h and electrophoresed in a 0.7% (w/v) TBE gel for 16 h at 25 V. DNA was processed further for Southern blotting as described (Sambrook *et al.*, 1989). Plasmid DNA was isolated from M5-1a strains and then electroporated into *E.coli* strain MH1 and analysed by restriction analysis.

PCR amplification and nucleotide sequencing

One purifed colony of a Ura^+ M5-1a strain was suspended in 50 μl H_2O and heated for 5 min at 95°C. After cooling on ice the lysate was centrifuged for 5 min at 15 000 r.p.m. An aliquot (5 μl) of this DNA suspension was added to 45 μl of reaction mixture [50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 200 μM each nucleotide, 0.5 μM each primer, 0.1 U *Taq* polymerase (Super *Taq*; HT Biotechnology Ltd, UK)]. The PCR reaction cycles were: 2 min at 95°C; 1 min at the annealing temperature; 2 min at 72°C; 30 cycles of 1 min at 95°C, 1 min at the annealing temperature and 2 min at 72°C; 1 min at the annealing temperature; 10 min at 72°C. The primers used in the PCR reaction were: p1 5′-CGTTGCGGTTCTGTCAGTTCC-3′ (annealing temperature 59°C); p2 5′-CGCCTTGCAGCACATCCC-3′ (annealing temperature 60°C); p3 5′-TCAACATGCTACCCTCC-3′ (annealing temperature 45°C). The PCR product (15 μl, ~200 ng DNA) was purified on an agarose gel in 1× TBE for 90 min at 70 V. The amplified fragment was cut out and the gel slice heated at 65°C for 10 min. To 5 μl of this suspension was added 5 μl H_2O, 2 μl primers (0.5–2 pmol) and 2 μl annealing buffer. This mixture was incubated for 10 min at 65°C and then solidified by cooling on ice. Labelling mix (3.5 μl) containing 0.5 μl [α-^{35}S]dATP (<37 Tbq/nmol, 37 mBq/100 μl), 1.6 μl T7 dilution buffer and 3 U T7 DNA polymerase was added to the annealing mix and incubated for 5 min at 40°C. Aliquots (3 μl) of the labelling reaction were added to 2.5 μl of each termination mix and incubated for 5 min at 40°C. The reaction was stopped by addition of 5 μl stop buffer. The samples were heated for 5 min at 95°C before loading and then electrophoresed on a 6% polyacrylamide gel for 2.5 h in 1× TBE at 2000 V and 35 mA. The gel was fixed in 20% (v/v) methanol and 10% acetic acid for 1 h. After drying at 85°C, autoradiography was for 3 days (Kodak XAR film).

Acknowledgements

We would like to thank Y.Steensma and T.Wenzel for their generous gifts of yeast strains and helpful advice. This work was supported by the Netherlands Foundation for Chemical Research (SON) and the Biotechnological and Biological Sciences Research Council (BBSRC), UK. Thanks are also due to Remko Offringa for critical reading of the manuscript.

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Received on January 16, 1995; revised on April 15, 1995